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SENSITIZATION OF MICE TO RICKETTSIAL TOXIN BY COXIELLA BURNETII

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Summary. — Intraperitoneal (ip.) inoculation with live or killed *Coxiella burnetii* (*C.b.*) rendered mice more sensitive to intravenous (iv.) administration of a toxic live suspension of *Rickettsia typhi*. Sensitization of mice by live and killed *C.b.* was time- and dose-dependent. Killed phase I and phase II *C.b.* cells possessed a similar degree of sensitization, which was increased slightly by their preincubation with corresponding immune sera. Lipopolysaccharide (LPS)-protein complex extracted from phase I *C.b.* cells exerted lower sensitization than whole phase I *C.b.* cells, and chloroform-methanol (CM) treatment of phase I *C.b.* cells reduced markedly their sensitizing effect. No toxic effect was observed either in *C.b.*-inoculated or in control mice upon i.v. administration of a heated *R. typhi* suspension. Specificity of rickettsial toxicity was demonstrated by its distinct reduction both in control and *C.b.*-inoculated mice after preincubation of *R. typhi* suspension with immune anti-*R. typhi* mouse serum.

Key words: Coxiella burnetii; sensitization; Rickettsia typhi; toxicity; mice

Introduction

During studies on the induction of nonspecific resistance in mice to various microorganisms by different *C.b.* preparations, a peculiar phenomenon was observed when *R. typhi* was used for challenge, namely that mice inoculated i.p. with *C.b.* died frequently within 24 hr after i.p. administration of *R. typhi*, though survivors remained protected against *R. typhi* infection (Kazár *et al.*, unpublished data). It suggested that *C.b.* preparation might sensitize mice to the toxic effect of *R. typhi*. To confirm this hypothesis, experiments were undertaken proving the sensitization of mice with *C.b.* to live toxic suspensions of *R. typhi*. The time and dose-dependence of the phenomenon, and the effects of preincubation of *C.b.* and *R. typhi*, respectively, with corresponding immune sera were studied.

Materials and Methods

R. typhi was grown in chick embryo (CE) yolk sacs. From moribund CE, the heavily infected yolk sac membranes (as determined by microscopic examination of Gimenez-stained preparations) were collected, their 40% suspension in brain-heart infusion (BHI) was prepared, sampled, frozen immediately and kept at −70 °C. The titre of *R. typhi* suspension assayed in yolk sac-inoculated CE and calculated according to Reed and Muench was $10^{8.6}$ EID_{50}/ml.

C. b. preparations. Pools of live *C.b.* Nine Mile strain in phase I (the 3rd egg passage — EP3) and in phase II (EP163 in our laboratory) prepared from infected CE yolk sacs and partially purified by differential centrifugation yielded the titres of $10^{8.1}$ and $10^{7.6}$ EID_{50}/ml, respectively. They served also for preparation of formalin-killed phase I or phase II *C.b.* cells purified as described (Schramek *et al.*, 1978). The purified *C.b.* cells were adjusted to the concentration of 2 mg/ml in phosphate buffered saline (PBS) solution and stored at −20 °C. In some experiments also phase I *C.b.* cells treated with a CM mixture as described (Kazár *et al.*, 1983) or the LPS-protein complex extracted from phase I *C.b.* cells by trichloracetic acid (TCA) according to Brezina and Úrvölgyi (1961) were used. The TCA-extracted LPS-protein complex was lyophilized, weighed and resuspended so that 1 ml of PBS contained 1 mg of the preparation.

R. typhi toxicity assay. R. typhi suspension diluted in BHI was kept on ice during the inoculation of fixed mice into the tail vein with 0.3 ml volumes of rickettsial dilution. Each dilution was tested on 4 mice (SPF mice from the VELAZ breed weighing 23—25 g) which were observed for 24 hr, when their death in each group was recorded.

Preparation of immune sera. Phase I and phase II immune sera to *C.b.* were collected 30 days after i.p. inoculation of rabbits with 10^6 EID_{50}/ml of phase I or phase II *C.b.* Nine Mile strain. Phase I serum contained antibodies directed to both antigen 1 and antigen 2 (in titres 64 and 256, respectively), phase II serum antibodies directed to antigen 2 only (titre of 128), as detected by complement-fixation (CF) test. Anti- *R. typhi* serum collected from mice infected for 2 weeks with about 10^5 EID_{50} of *R. typhi* had CF antibody titre of 128 with corpuscular *R. typhi* antigen.

Results

Determination of toxicity of R. typhi suspension

Mice were inoculated i.v. with increasing dilutions (1 : 5, 1 : 10, 1 : 15, 1 : 20, 1 : 25, 1 : 30, and 1 : 40) of *R. typhi* suspension and toxic deaths were recorded. In repeated experiments, the deaths occurred within 24 hr post inoculation (p.i.), the LD_{50} dose being present in the dilution of 1 : 20 of the rickettsial suspension tested.

Table 1. Time -dependence of sensitization of mice to R. typhi toxin by killed phase I C. burnetii cells

Days after *C.b.* inoculation*	Administration of *R. typhi* suspension diluted				
	1 : 20	1 : 40	1 : 60	1 : 80	1: 120
1	3**	2	n.d.	0	
3	4	4	n.d.	1	0
7	4	4	4	3	0
14	n.d.	4	3	2	1
21	n.d.	4	n.d.	3	1
Controls	2	0	0		

* 1000 μg of *C.b.* was administered

** Number of dead mice out 4 mice tested. n.d. = not done

Table 2. Dose-dependence of sensitization of mice to R. typhi toxin by killed phase I C. burnetii cells

Dose of phase I *C.b.* cells* (μg)	Administration of *R. typhi* suspension diluted 1 : 20	1 : 40	1 : 60	1 : 80	1 : 120
1000	n.d.	4	4	3	0
100	4	4	4	1	0
10	4**	1	1	0	
Controls	1	0	0		

* Given 7 days before *R. typhi*.
** Number of dead mice out 4 mice tested. n.d. = not done

Time-dependence of sensitization of mice to R. typhi toxin by killed phase I C.b. cells

Mice were inoculated i.v. with increasing dilutions of *R. typhi* suspension on days 1, 3, 7, 14 and 21 after i.p. administration of 1000 μg of phase I killed *C.b.* cells. Non-inoculated mice served as controls. As shown in Table 1, phase I killed *C.b.* cells sensitized mice to the toxic effect of *R. typhi* as early as from the 1st day p.i.

This sensitizing effect was higher when prolonging the interval between *C.b.* and *R. typhi* administrations up to 7 days. Because no substantial difference in sensitization was observed when the interval was prolonged to 14 and 21 days, in all further experiments *R. typhi* was inoculated 7 days after administration of killed *C.b.* preparations.

Dose-dependence of sensitization of mice to R. typhi toxin by killed phase I C.b. cells

Mice inoculated i.p. with decreasing amounts of phase I killed *C.b.* cells (1000, 100, and 10 μg) were given i.v. on day 7 the increasing dilutions of *R. typhi* suspension. Table 2 shows that the higher was the dose of *C.b.* the higher was also the degree of sensitization to *R. typhi* toxin. Even the dose of 10 μg of *C.b.* exerted a clear sensitizing effect.

Table 3. Comparison of sensitizing ability to R. typhi toxin of diferent killed C. burnetii preparations

C.b. preparation used*	1 : 20	1 : 40	1 : 60	1 : 80	1 : 120	1 : 160
Phase I cells untreated		4	4	2	1	0
Phase I cells CM-treated	2**	1	0	0		
Phase I cells TCA-extract	4	1	1	0		
Phase II cells	4	4	3	3	1	0
Controls	2	0				

* 1000 μg was administered with the exception of TCA-extract (200 μg) 7 days before *R. typhi* inoculation
** Number of dead mice out 4 mice tested.

Table 4. The effect of preincubation of phase I and phase II killed C. burnetii cells with corresponding immune sera on their sensitizing effect to R. typhi toxin

C.b. preparation used*	Administration of *R. typhi* suspension diluted			
	1 : 20	1 : 40	1 : 80	1 : 160
Phase I cells + normal serum	4**	4	1	1
Phase I cells + immune serum	4	4	3	1
Phase II cells + normal serum	4	4	1	0
Phase II cells + immune serum	4	4	4	1
Controls	3	0		

* 500 μg was preincubated for 1 hr at 37 °C with the serum given 7 days before *R. typhi* inoculation.
** Number of dead mice out 4 mice tested.

Comparison of senziting ability to R. typhi toxin of different killed C.b. preparations

Mice were inoculated i.p. with 1000 μg of phase I or phase II killed *C.b.* cells, with 1000 μg of CM-treated phase I killed cells or with 200 μg of TCA-extracted LPS-protein complex. Seven days later they were inoculated i.v. with dilutions of the toxic *R. typhi* suspension. As follows from Table 3, the degree of sensitization to *R. typhi* toxin afforded by phase I and phase II killed *C.b.* cells was similar, toxicity being recorded in *R. typhi* dilutions 4 times higher than in control mice. Lower degree of sensitization was found in mice inoculated with the TCA-extracted LPS-protein complex, and treatment of phase I killed *C.b.* cells with a CM mixture almost completely abolished their sensitizing effect.

The effect of preincubation of phase I and phase II killed C.b. cells with corresponding immune sera on their sensitizing effect to R. typhi toxin

Phase I and phase II killed *C.b.* cells in concentrations 2 mg/ml (2000 μg) were mixed with the same volumes of corresponding rabbit phase I or phase II serum diluted 1 : 4 and with similarly diluted normal rabbit serum, respectively. After 1 hr incubation at 37 °C, 0.5 ml aliquots of mixtures (containing 500 μg of *C.b.* cells) were inoculated i.p. into mice which were given i.v. 7 days later dilutions of toxic *R. typhi* suspension. The results from Table 4 indicate that preincubation of both phase I and phase II *C.b.* cells with corresponding immune sera did not reduced, but rather slighty increased their sensitizing effect to *R. typhi* toxin.

Characterization of toxicity of R. typhi and its specificity

Since the rickettsial toxins are thermolabile their activivy being connected with live rickettsiae, one experiment was designed so that control mice and those inoculated with 1000 μg of phase I killed *C.b.* cells were injected i.v.

Table 5. The effect of preincubation of R. typhi with immune serum on its toxicity in C. burnetii--inoculated mice

C.b. preparation used*	Administration of *R. typhi* suspension diluted					
	10S[a]	20S[a]	40S[a]	10	20	40
Phase I killed cells	2**	0	0	n.d.	4	4
Controls	0	0	n.d.	4	0	n.d.

* 1000 μg was inoculated 7 days before *R. typhi* administration.
** Number of dead mice out 4 mice tested.
[a] S refers to preincubation with anti-*R. typhi* serum for 30 min at 37 °C.
n.d. — not done.

after 7 days with double dilutions (from 1 : 5 to 1 : 40) of *R. typhi* suspension which was heated for 30 min to 80 °C or kept for the same time on ice. Whereas the latter suspension was toxic as expected up to dilution 1 : 20 and 1 : 80 in control and *C.b.* -inoculated mice, respectively, heated *R. typhi* suspension was nontoxic in either group of mice, thus confirming thermolability of the toxic principle and its association with live rickettsiae.

To determine the specificity of *R. typhi* toxic effect, control mice and those inoculated i.p. with 1000 μg of phase I killed *C.b.* cells were given i.v. 7 days later dilutions of *R. typhi* toxic suspensions which were preincubated for 30 min at 37 °C with the same volumes of anti-*R. typhi* immune or control mouse sera (diluted 1:4). Preincubation of *R. typhi* with immune anti-*R. typhi* serum reduced completely its toxicity in control mice and distinctly in *C. b.* -inoculated mice, indicating that the toxicity of *R. typhi* was specific in either group of mice tested (Table 5).

Table 6. Development of sensitization of mice infected with different doses of C. burnetii to R. typhi toxin

Dose and days of *C.b.* infection*		Administration of *R. typhi* suspension diluted			
		1 : 15	1 : 30	1 : 60	1 : 120
10^7	1 day	4**	4	4	0
10^4	3 days	4	4	1	0
10^7	7 days	4	4	3	0
10^4	7 days	4	4	2	0
10^1	7 days	4	4	1	0
10^7	21 days	4	4	4	2
10^4	21 days	4	4	4	1
10^1	21 days	4	4	1	0
Controls		4	1	0	

* Mice infected with a given EID_{50} of phase I virulent strain.
** Number of dead mice out 4 mice tested.

Development of sensitization of mice infected with different doses of C.b. to R. typhi toxin

To find out whether susceptibility of mice to *R. typhi* toxin can be increased also by *C.b.* infected i.p. with three different doses (10^7, 10^4, and 10^1 EID_{50}) of phase I virulent *C.b.* strain were inoculated i.v. at intervals (from 1 to 21 days p.i.) with dilutions of *R. typhi* toxic suspension.

As follows from Table 6, phase I live *C.b.* cells induced also sensitization of mice to *R. typhi* toxin which was time and dose-dependent. Sensitizing effect was observed as early as one day p.i. when the highest infectious dose (10^7 EID_{50}) of *C.b.* was used. At later intervals (7 and 21 days post-infection) the degree of sensitization by doses of 10^7 and 10^4 EID_{50} was similar; that induced by the lowest infectious dose of 10^1 EID_{50} of *C.b.* was lower, but still present.

Discussion

Recently accumulated data on the ability of *C.b.* cells or their extracts to induce in experimental animals non-specific resistance to unrelated microorganisms (Kelly, 1977; Paquet *et al.*, 1979; Clark, 1979) and tumours (Kelly *et al.*, 1976; Kazár and Schramek, 1979) suggest that *C.b.* can be included into the group of microorganisms possessing immunomodulatory properties. The mechanisms of nonspecific resistance afforded by *C.b.* are not known as yet, though activation of macrophages (Kelly, 1977), induction of interferon (Kazár, 1966; Brezina *et al.*, 1968), stimulation of natural killer cells (Clark, 1979), splenomegaly with stimulation of mononuclear phagocytic system (Kazár *et al.*, 1983) may explain some *C.b.*-induced effects.

Besides these effects which might be considered as favourable for the hosts, *C.b.* can modify the immune competence also in the negative sense, i.e. by suppressing the normal response of lymphocytes to mitogens (Kishimoto and Gonder, 1979; Damrow *et al.*, 1981), and adverse effects of *C.b.* namely of corpuscular vaccines, such as severe local reactions (Luoto *et al.*, 1963) and occasional systemic reactions (Anacker *et al.*, 1962) are notoriously known. Studies carried out in our laboratory showed that *C.b.* was able to induce hyperreactivity of mice to bacterial endotoxin (Schramek *et al.*, to be published) and as evidences by this our study to sensitize mice to the toxic effect of *R. typhi*.

Senzitization of mice to rickettsial toxin was time-dependent at least at earlier intervals both by live and killed phase I *C.b.* cells. At later intervals (from 7 to 21 days), however, differences in the degree of sensitization by killed *C.b.* cells were not so high and after inoculation of live *C.b.* cells the degree of sensitization by two different infectious doses (10^7 and 10^4 EID_{50}) was quite similar, suggesting that in the latter case a certain accumulation of *C.b.* cells in the mouse viscera was necessary. This was confirmed also by dependence of the degree of sensitization of the dose of phase I killed *C.b.* cells. Splenomegaly of mice resulting from the inoculation of live (Kazár *et al.*, 1982) or killed *C.b.* cells (Kazár *et al.*, 1983) was also time- and dose-

-dependent, reaching the maximum in about 3 weeks after inoculation. Its development along with hepatomegaly seems to be decisive for the induction by *C.b.* cells of hyperreactivity in mice to bacterial endotoxin (Schramek *et al.*, to be published). It is possible that similar changes in the mouse spleen and/or liver, such as an accumulation of macrophages in the spleen from which pharmacologically active mediators can be released (Michalek *et al.*, 1980) or a disturbance of detoxification function of the liver, in which *C.b.* experimental infection induces different metabolic changes (Paretsky and Stueckemann, 1970; Thompson and Paretsky, 1973; Heggers *et al.*, 1975; Marecki *et al.*, 1978) and which is involved in acute (Pellegrin *et al.*, 1980) and chronic (Turck *et al.*, 1976) human Q fever infection, are responsible for sensitization of mice to both bacterial endotoxin and rickettsial toxin. On the other hand, some differences between sensitization to bacterial endotoxin and rickettsial toxin as concerns phase II *C.b.* cells-sensitizing ability or differences in sensitization by phase I killed *C.b.* cells after their preincubation with phase I immune serum, indicate that in the induction of sensitization to bacterial endotoxin and rickettsial toxin different mechanisms, cells or mediators influencing on the permeability of capillaries by an injury of the endothelial cells can be involved. In this connection also the fact that *R. typhi* toxin is thermolabile, and its activity is associated with live, metabolic active rickettsiae (Cooke, 1961), must be taken into account.

Of importance is the observation of lower sensitization to rickettsial toxin by TCA-extract from phase I cells, which was recommended (Brezina *et al.*, 1974) and successfuly employed (Kazár *et al.*, 1982) as Q fever chemovaccine for human use. Similarly, the CM-treatment of phase I killed *C.b.* cells reduced markedly their sensitizing effect not only to rickettsial toxin, but also to bacterial *endotoxin* (Schramek *et al.*, in preparation), suggesting the suitability of the use of CM-treated instead of untreated phase I killed *C.b.* cells for field vaccination of domestic animals, providing that CM-treated and untreated vaccines afford the comparable level of protection, as found before (Wiliams and Cantrell, 1982; Kazár *et al.*, 1983). From results with the CM-treatment of phase I cells follows an assumption that for sensitization to toxic substances phospolipids of the cell-wall of *C.b.* can be responsible. More detailed knowledge on the relationship between structure and function of *C.b.* components, which is the purpose of our contemporary and future studies will be helpful in improving both diagnosis and specific prevention of Q fever.

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